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Report

MeCP2-Dependent Transcriptional Repression Regulates Excitatory Neurotransmission

Erika D. Nelson,[1] Ege T. Kavalali,[2] and Lisa M. Monteggia[1,*]
[1] Department of Psychiatry
[2] Center for Basic Neuroscience
University of Texas Southwestern Medical Center
Dallas, Texas 75390

Summary

Mutations in the transcriptional repressor, methyl-CpG binding protein 2 (MeCP2), result in a neurodevelopmental disorder called Rett Syndrome (RTT) [1–5]. Based on the neurological phenotypes observed in Rett patients, we examined the potential role of MeCP2 in synaptic function. We compared elementary properties of synaptic transmission between cultured hippocampal neurons from MeCP2 knockout and wild-type littermate control mice and found a decrease in the frequency of spontaneous excitatory synaptic transmission (mEPSCs) in neurons lacking MeCP2. We also detected a significant increase in the rate of short-term synaptic depression. To explore whether these functional effects can be attributed to MeCP2's role as a transcriptional silencer, we treated cultures with a drug that impairs histone deacetylation and examined spontaneous synaptic transmission. Treatment with this compound induced a similar decrease in mEPSC frequency in wild-type control cultures, but this decrease was occluded in MeCP2-deficient neurons. Interestingly, neither the loss of MeCP2 nor the drug treatment resulted in changes in mIPSC properties. Finally, by means of a lentivirus expressing Cre recombinase, we show that loss of MeCP2 function after neurodevelopment and synaptogenesis was sufficient to mimic the decrease in mEPSC frequency seen in constitutive MeCP2 KO neurons. Taken together, these results suggest a role for MeCP2 in control of excitatory presynaptic function through regulation of gene expression.

Results

Decrease in the Frequency of mEPSCs in MeCP2 Knockout Neurons

To elucidate the role of MeCP2 in the regulation of synaptic transmission, we studied functional alterations of synapses in 11–14 days in vitro hippocampal cultures made from newly born MeCP2 KO mice. Recent electrophysiological measurements of synaptic plasticity in MeCP2-deficient mice were performed on both hippocampal and cortical slices [6, 7]. Dissociated primary cultures allow examination of synaptic function independent of potential general alterations in brain homeostasis, thereby enabling a distinction between cell-autonomous defects and global systemic dysfunction. We quantified the frequency and amplitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) in MeCP2 KO and wild-type (wt) littermate control cultures by means of whole-cell recordings performed in the presence of tetrodotoxin (TTX) to block action potential firing and picrotoxin to block inhibitory activity. We found a significant decrease in the frequency of mEPSCs in the KO neurons compared to wt controls (Figures 1A–1C). The decrease was also observed in older cultures (>20 DIV; data not shown), suggesting that the loss of MeCP2 produces long-term alterations in excitatory synaptic transmission. This alteration in mEPSC frequency may implicate a presynaptic deficit in the MeCP2 KO neurons. In contrast, the frequency of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) was unaffected, suggesting a specificity of MeCP2 function in excitatory neurotransmission (Figures 1E–1G). The amplitudes of individual synaptic events in both mEPSCs and mIPSCs were also unaffected by the loss of MeCP2, indicating no change in the number of postsynaptic receptors at either excitatory or inhibitory synapses (Figures 1D and 1H).

To better understand how the loss of MeCP2 may contribute to the alteration in mEPSC frequency, we examined the number of presynaptic terminals formed on pyramidal neuron dendrites in culture. Neurons were immunostained for microtubule-associated protein (MAP2) and synapsin, a synaptic vesicle protein, to identify presynaptic terminals on the dendrites and soma. This analysis revealed that the number of presynaptic terminals was unchanged in MeCP2 KO neurons compared with wt neurons, suggesting that the decrease in spontaneous synaptic events is not the result of a decreased number of presynaptic terminals (Figures 1I and 1J).

Characterization of Spontaneous and Evoked Neurotransmission in MeCP2-Deficient Cultures

We next examined whether there was a decrease in the size of the total recycling pool and the number of readily releasable vesicles (see Figure S1 in the Supplemental Data available with this article online). To probe the total pool size, we stimulated cultures with 47 mM K^+ solution for 90 s in the presence of the styryl dye FM1-43 [8]. This strong stimulation normally labels all recycling vesicles within a presynaptic terminal [9]. After dye wash out (~10 min), we stimulated the cultures with 90 mM K^+ solution applied four times, the first for 90 s followed by three applications of 60 s each (each separated by 60 s intervals), to release all the dye trapped within presynaptic terminals. The kinetics of dye loss from synaptic terminals (Figure S1A) and the total amount of dye trapped in individual synapses was indistinguishable between KO and control neurons (Figure S1B), indicating that the sizes of the total vesicle pools were unchanged. To quantify differences in the number of readily releasable vesicles, we stimulated cultures with a brief hypertonic sucrose application, which selectively releases vesicles

*Correspondence: lisa.monteggia@utsouthwestern.edu

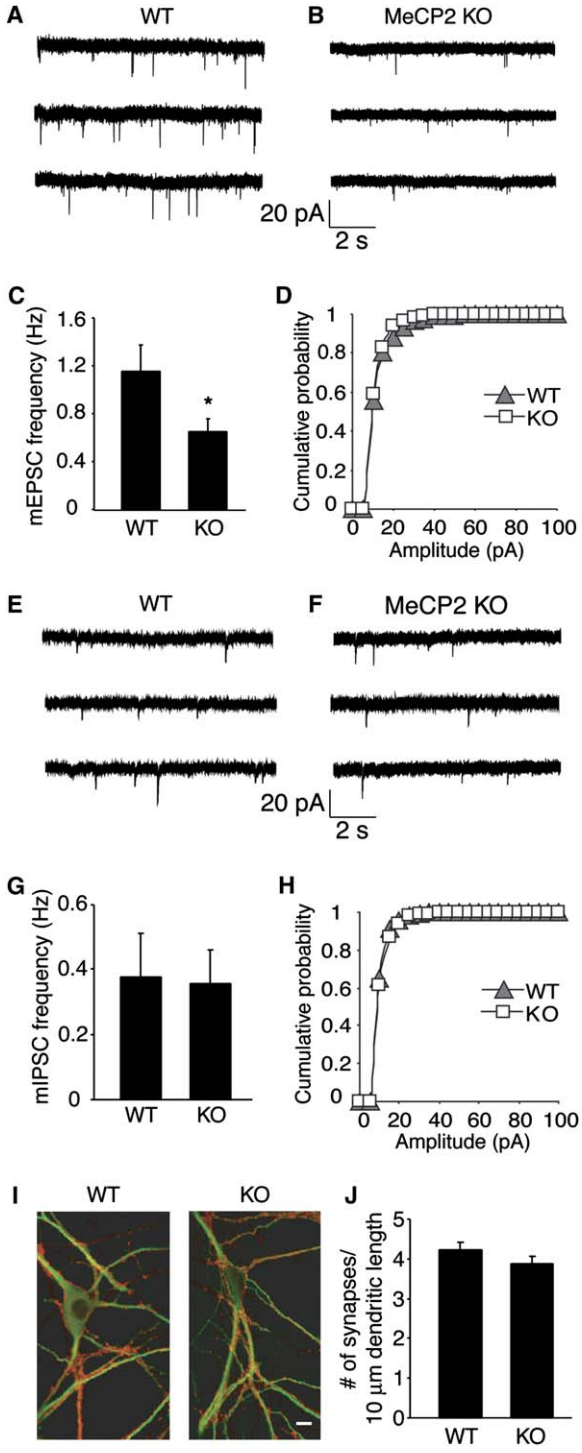

Figure 1. Spontaneous Miniature Synaptic Currents in Cultured Hippocampal Neurons from MeCP2 Knockout and Control Mice

(A–D) Spontaneous excitatory synaptic currents. Representative recordings of miniature excitatory events in control (A) and MeCP2 knockout (B) neurons recorded in 1 μM TTX and 50 μM picrotoxin. (C) Bar graph showing a decrease in the frequency of spontaneous excitatory events in knockout neurons compared to controls (*$p < 0.05$). (D) Cumulative histogram of mEPSC amplitudes.

(E–H) Spontaneous inhibitory synaptic currents. Representative recordings of miniature inhibitory events in control (E) and MeCP2 knockout (F) neurons recorded in 1 μM TTX and 10 μM NBQX. (G) Bar graph showing similar frequencies of spontaneous inhibitory events in control and knockout neurons. (H) Cumulative histogram of mIPSC amplitudes.

(I and J) Immunostaining of cultured neurons. (I) Dissociated neurons from control and knockout mice were labeled with primary antibodies to MAP2 (green) and Synapsin (red). (J) Bar graph depicts the number of presynaptic terminals found in control and mutant neurons. There was no significant difference in the number of presynaptic terminals between control and MeCP2 knockout neurons ($p > 0.2$).

Error bars show standard error of the mean.

from the readily releasable pool [10]. We found no difference between the MeCP2 KO and wt neurons, suggesting that the changes in spontaneous release frequency in the KO cultures cannot be attributed to a reduction in the number of readily releasable vesicles (Figures S1C and S1D). However, we cannot fully exclude the possibility that the numbers of spontaneously recycling vesicles are reduced, but we consider this unlikely given that sizes of distinct vesicle pools are usually highly correlated in central synapses [11].

We next examined the properties of evoked neurotransmission in response to action potential stimulation. Trains of action potentials applied at 10 Hz typically depress neurotransmission. This depression is, at least in part, elicited by a rapid decrease in the number of vesicles available for release within a synaptic terminal. When the kinetics of synaptic depression and recovery in the MeCP2 KO cultures were examined, we observed a more rapid depression during 10 Hz stimulation and a slower response recovery when the stimulation frequency was switched to 1 Hz at the end of the 10 Hz train compared to wt cultures (Figures 2A, 2C, and 2D). The KO cultures also showed a slight increase in first response amplitudes at 10 Hz and significantly smaller paired pulse ratios during high-stimulation frequencies compared to controls (Figures 2B, 2C, and insert). These findings suggest that the loss of MeCP2 may contribute to an increase in neurotransmitter release probability leading to a faster depletion of releasable vesicles, as well as a delay in synaptic vesicle recycling after stimulation retarding the recovery of responses. Taken together with the decrease in spontaneous event frequency, these findings suggest a role for MeCP2 in presynaptic control of neurotransmitter release and vesicle recycling.

Inhibition of Transcriptional Silencing Decreases mEPSC Frequency

To examine whether the alterations in excitatory neurotransmission of neurons lacking MeCP2 are the result of impairments in gene silencing, we treated wild-type C57BL/6 hippocampal cultures chronically with drugs that inhibit either transcriptional activation or repression and then measured synaptic transmission. To suppress transcription, we treated cultures with the RNA polymerase inhibitor Actinomycin-D. To suppress transcriptional repression, we used Trichostatin A (TSA), a histone deacetylase inhibitor. Cultures were treated with these individual drugs (and DMSO as a control) for 24 hr and then synaptic activity was measured. We preferred chronic rather than acute treatments because earlier studies did not reveal a significant change in baseline synaptic

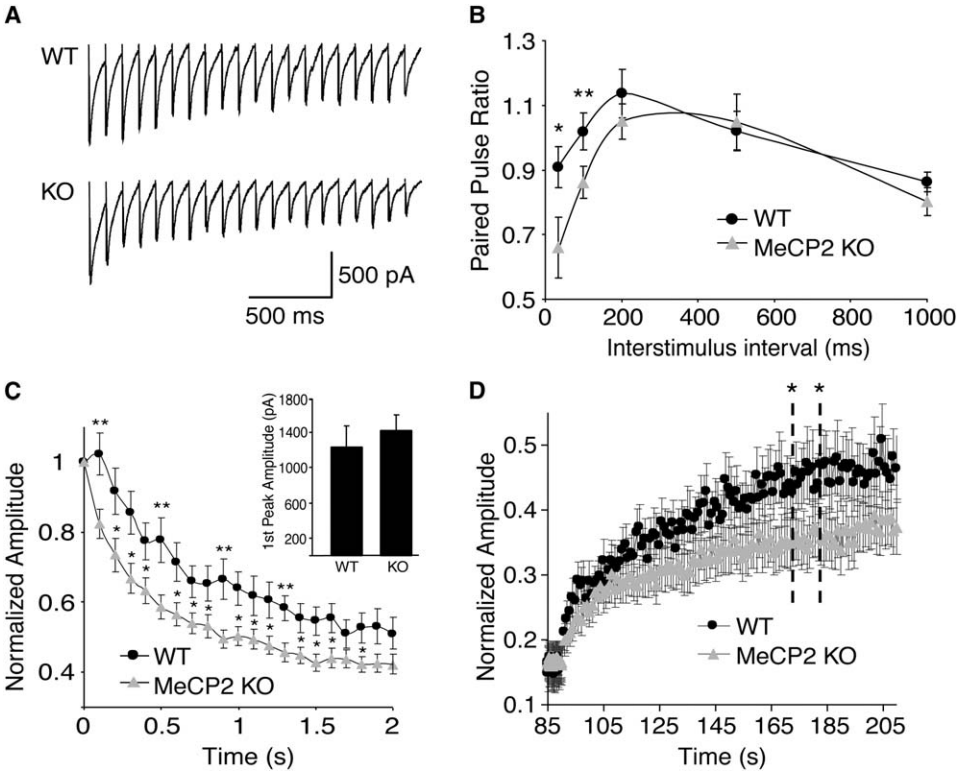

Figure 2. Evoked Synaptic Responses in MeCP2 Knockout and Control Neurons during 10 Hz Field Stimulation Immediately Followed by 1 Hz Recovery Stimulation

(A) Representative whole-cell recordings of the first 20 responses.

(B) Paired pulse ratios of the first two responses during various stimulation frequencies were measured from control and knockout neurons. The paired pulse ratios recorded from MeCP2 knockout neurons were significantly less than control at both 30 Hz and 10 Hz frequencies (*$p < 0.05$; **$p < 0.01$).

(C) Average response amplitudes of control and mutant neurons measured during the first 2 s of 10 Hz stimulation. Recorded responses from field stimulated knockout neurons depressed significantly faster than control responses (*$p < 0.05$; **$p < 0.01$). Insert is a bar graph depicting similar average first peak amplitudes of these responses.

(D) Average response amplitudes measured during 1 Hz stimulation following the 10 Hz depression. Knockout response amplitudes did not recover as quickly as those recorded from control neurons (*$p < 0.05$).

Error bars show standard error of the mean.

transmission after acute TSA application, although they reported a strong augmentation of long-term synaptic plasticity [12]. We initially examined cell viability in the presence of these chronic treatments and found that it was not compromised (see Experimental Procedures). In these experiments, we focused on potential alterations in the frequency and amplitude of spontaneous miniature events, which is a more direct measurement of presynaptic machinery, since evoked transmission may be vulnerable to alterations in membrane excitability and Ca^{2+} signaling, two aspects of neuronal function that may also be targeted by transcriptional regulation. We found a significant decrease in mEPSC frequency in TSA-treated cultures compared to DMSO-treated cultures, while Actinomycin-D did not have an effect (Figures 3A and 3B). The average amplitudes of individual events were not significantly affected by these treatments (data not shown). We also determined the number of presynaptic terminals in these cultures and found no changes between the control and TSA treatment (Figures 3C and 3D). Similarly to what was seen with the MeCP2 KO cultures, both the frequency and amplitudes of mIPSCs were unchanged between the DMSO- and TSA-treated neurons (Figures 3E and 3F and data not shown). To investigate whether newly transcribed genes are involved in the suppression of synaptic function, we treated cultures with both Actinomycin-D and TSA and found a reversal of the mEPSC deficits seen in the TSA-treated neurons (Figure 3B). These findings indicate a selective impairment of excitatory presynaptic function after suppression of transcriptional repression but not transcriptional activation.

To examine whether the TSA-mediated decrease in mEPSCs was related to MeCP2 function, we chronically treated MeCP2 KO cultures with TSA and did not detect a reduction in the frequency of spontaneous mEPSCs (Figures 3G and 3H). This result strongly suggests that the decrease in spontaneous miniature frequency we observed in the presence of this transcriptional activator was in significant part due to inhibition of MeCP2 function and thus was occluded in the absence of MeCP2. Interestingly, Actinomycin-D did not rescue the reduction in mEPSC frequency seen in MeCP2 KOs back to control levels (Figures 3G and 3H). This may not be surprising since degradation of abnormally expressed presynaptic proteins may take longer than 24 hr.

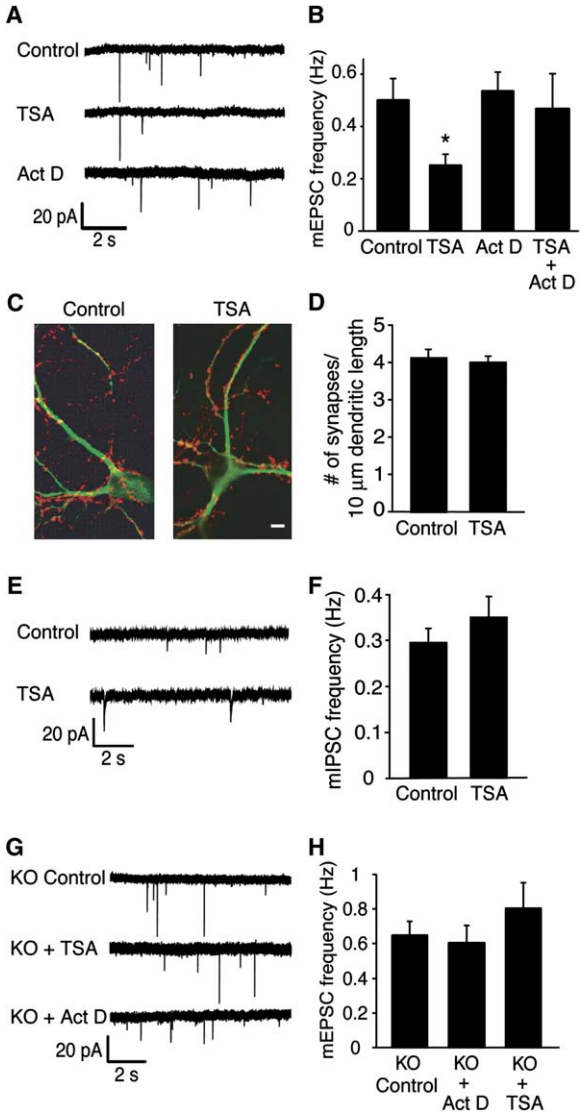

Figure 3. Spontaneous Miniature Synaptic Responses from Wild-Type C57BL/6 and MeCP2 Knockout Hippocampal Cultures after a 24 hr Treatment with Inhibitors of Transcriptional Repression and Activation

(A and B) Spontaneous excitatory synaptic currents from C57BL/6 neurons. (A) Representative miniature excitatory synaptic currents from drug-treated neurons recorded in 1 μM TTX and 50 μM picrotoxin. (B) Bar graph of the significant decrease in mEPSC frequencies in neurons treated for 24 hr with the inhibitor of transcriptional repression, TSA (*$p < 0.05$). This decrease was reversed when treatment included both TSA and the inhibitor of transcriptional activation, Act D.

(C and D) Immunostaining of C57BL/6 cultured neurons. (C) Control and TSA-treated dissociated neurons were labeled with primary antibodies to MAP2 (green) and Synapsin (red). (D) Bar graph depicting a similar number of presynaptic terminals found in control and TSA-treated neurons.

(E and F) Spontaneous inhibitory synaptic currents from C57BL/6 neurons. (E) Representative recordings of miniature inhibitory events from control and TSA-treated neurons recorded in 1 μM TTX and 10 μM NBQX. (F) Bar graph showing the frequencies of spontaneous miniature inhibitory events.

(G and H) Spontaneous excitatory synaptic currents from MeCP2 knockout neurons. (G) Representative traces from drug-treated knockout neurons recorded in the presence of 1 μM TTX and 50 μM picrotoxin. (H) Bar graph showing that mEPSC frequencies in knockout neurons were not significantly affected by 24 hr treatment with either Act D or TSA. Error bars show standard error of the mean.

Alterations in mEPSC Frequency in Floxed MeCP2 Neurons Infected with a Lentivirus Expressing Cre Recombinase

We also investigated whether an acute loss of MeCP2 function after neurodevelopment and synapse maturation would result in similar phenotypes found in the constitutive MeCP2 KO neurons. We made primary dissociated hippocampal cultures from newborn floxed MeCP2 mice and allowed them to age 7 days in vitro before infecting them with high or low titer lentivirus expressing the gene Cre recombinase, or GFP as a control. One week later, we found a significant decrease in the frequency of mEPSCs in high titer Cre-infected floxed MeCP2 neurons compared to GFP-infected neurons (Figures 4A and 4B). This significant frequency decrease was also seen when mEPSCs from Cre-infected floxed MeCP2 neurons were compared with Cre-infected wild-type littermate neurons, ruling out a nonspecific effect of Cre expression on mEPSCs (Figures 4A and 4B). These data suggest that MeCP2 acts as a regulator of synaptic transmission even in mature neurons and the loss of MeCP2 may have profound effects on synaptic function after neurodevelopment. We also recorded mEPSCs from uninfected floxed MeCP2 neurons receiving the majority of their inputs from Cre-infected neurons and found the same decrease in event frequency supporting the presynaptic origin of this observation (Figures 4A and 4B). In accordance with this premise, mEPSC frequency was not altered in floxed MeCP2 neurons infected with a low titer Cre-expressing lentivirus compared to GFP controls (Figures 4D and 4E). These data, as well as the fact that we saw no significant changes in mEPSC amplitudes among any of the lentiviral infected cultures (Figures 4C and 4F), strongly suggest that MeCP2 plays a specific role in presynaptic function.

Discussion

Taken together, these findings suggest that synaptic transmission, in particular presynaptic function, is under transcriptional control and that MeCP2-dependent transcriptional repression is a critical component of this regulation. In our experiments, we detected MeCP2-dependent alterations in spontaneous neurotransmission as well as in short-term synaptic depression. Spontaneous neurotransmission is important for a number of neuronal processes, which include maturation and stability of synaptic networks [13, 14] and inhibition of local dendritic protein synthesis [15]. Therefore, the decrease in mEPSCs we describe here may well underlie some of the neuromorphological abnormalities seen in RTT patients as well as RTT mouse models [16–19]. However, our data also suggest genes involved in evoked transmission and short-term plasticity as potential transcriptional targets under MeCP2 control. Short-term synaptic depression is a fundamental synaptic mechanism, which underlies key brain functions such as sound localization and sensory adaptation [20–22]. Therefore, changes in synaptic depression may have important implications for the synaptic basis of RTT as well as other neurodevelopmental disorders.

Recent evidence suggests that synaptic vesicles giving rise to evoked and spontaneous neurotransmission originate from different pools [11]. Therefore, it is

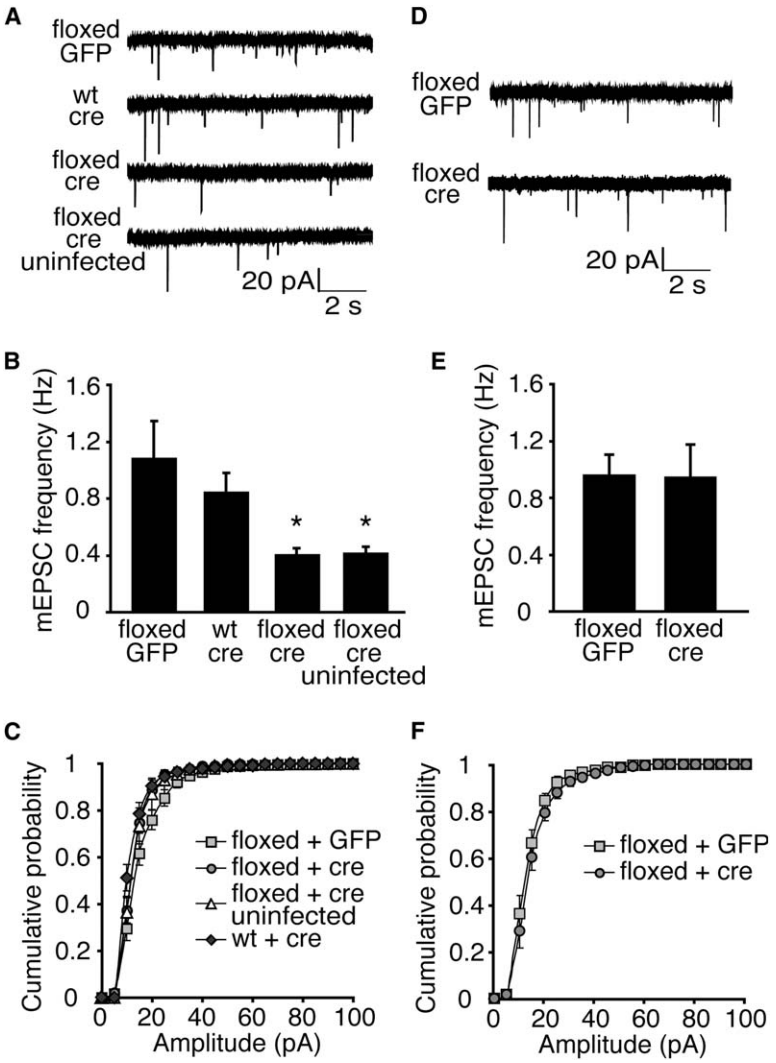

Figure 4. Spontaneous Miniature Excitatory Synaptic Currents in Floxed MeCP2 Neurons Infected with a Lentivirus Expressing Cre Recombinase

(A–C) Miniature excitatory events from floxed neurons infected with high titer lentivirus. (A) Representative traces from infected neurons recorded in the presence of 1 μM TTX and 50 μM picrotoxin. (B) Bar graph revealing a significant decrease in mEPSC frequencies from floxed MeCP2 neurons infected after synapse formation with high titer lentiviral Cre recombinase (*$p < 0.05$). This decrease was seen in recordings from both Cre-infected and uninfected postsynaptic floxed neurons and is compared to both floxed neurons infected with GFP and wild-type neurons infected with Cre. (C) Cumulative histogram showing no significant changes in mEPSC amplitudes.

(D–F) Miniature excitatory currents from floxed neurons infected with low titer lentivirus. (D) Representative traces of mEPSCs recorded from infected neurons. (E) Bar graph revealing similar mEPSC frequencies from floxed MeCP2 neurons infected after synapse formation with low titer lentiviral Cre recombinase (*$p < 0.05$). (F) Cumulative histogram showing no significant changes in mEPSC amplitudes.

Error bars show standard error of the mean.

possible to envision that simultaneous changes in expression levels of multiple synaptic proteins, which are under MeCP2 regulation, may have opposing effects (or a complex additive effect) on the two forms of neurotransmission. Understanding the mechanisms underlying these synaptic alterations will require identification of synaptic molecules that are MeCP2 dependent in their transcription.

Could the alterations in synaptic transmission produced by mutations in the MeCP2 gene underlie the behavioral phenotypes observed in RTT patients? Recent studies have shown that the loss of MeCP2 selectively in the brain is sufficient to recapitulate many features of RTT, including impaired motor coordination, increased anxiety-related behavior, and social deficits [23]. In related studies, brain slices from mice overexpressing MeCP2 displayed an increase in paired pulse facilitation and long-term potentiation (LTP) [24], while MeCP2 null mice exhibited the converse [7], suggesting that MeCP2 expression may exert a profound role on synaptic plasticity. Our data indicate a specific deficit in excitatory synaptic transmission upon the loss of MeCP2 function. A recent study of cortical slices from 4- to 5-week-old MeCP2 knockout mice also found changes in excitatory, as well as inhibitory, transmission [6]. These additional inhibitory changes may be specific for cortical neurons, or they may just occur later in development. Nevertheless, it appears that an imbalance in excitatory and inhibitory input as we see in hippocampal cultures may underlie some of the neurological deficits in RTT, and possibly other related neurodevelopmental disorders. Indeed, other recent studies have shown that the neuroligin genes, a family of proteins implicated in autism spectrum disorders, are important for maintaining a balance of excitatory and inhibitory neurotransmission [25, 26].

It is worthwhile noting that we did not observe any morphological changes in neurons lacking MeCP2. This may well be due to the fact that our studies were done in young cultures and may reflect a more immature neuronal population. This suggests that the functional deficits we observed in MeCP2 KO neurons may preclude the neuronal structural abnormalities observed in the disease state.

These data suggest that transcriptional repression is important in regulating presynaptic function of hippocampal neurons. This provides insight into how the loss of function of the transcriptional repressor, MeCP2, may

contribute to the disease state and may have profound implications, especially if additional neuronal populations are under MeCP2 control. This information is important in delineating the cellular and functional abnormalities that lead to the wide array of neurological deficits observed in RTT patients.

Experimental Procedures

Cell Culture

Whole hippocampi were dissected from the brains of 1-day-old MeCP2 null knockout mice (Jackson Laboratories), floxed MeCP2 mice, or littermate controls, and dissociated cultures were prepared according to previously published protocols [27]. For drug treatment experiments, whole hippocampi were dissected from the brains of 1-day-old C57BL/6 mice and cultures prepared under identical conditions. All experiments were performed on cultures 11–14 days in vitro (DIV).

Drug Treatments and Cell Viability

Hippocampal cultures were treated for 24 hr at 10–11 DIV with the following drugs: dimethyl sulfoxide (DMSO) (1:1000), Trichostatin A (TSA) (1 μM), or Actinomycin D (Act D) (2.5 μM). After drug treatments, cell viability was checked by Trypan Blue exclusion (Sigma). The percentages of dead cells were not significantly different between control cultures (6.8% ± 1.8% SEM, DMSO-treated) and cultures treated with drugs (6.8% ± 1.1%, TSA, and 3.5% ± 1.8%, ActD).

Immunocytochemistry

Dissociated hippocampal neurons were fixed for 30 min with 4% paraformaldehyde, rinsed twice with 1× PBS/Glycine, then blocked in 2% goat serum for 1 hr. The cells were then incubated with primary antibodies, anti-MAP2 monoclonal (1:200, Chemicon), and anti-synapsin polyclonal (1:1000, Synaptic Systems) overnight at 4ºC. The next day the cells were washed, then incubated with fluorescent secondary antibodies, goat-anti-rabbit (1:200, Molecular Probes), and goat-anti-mouse (1:200, Molecular Probes). Coverslips were mounted with Vectashield (Vector Laboratories) and neurons were visualized on a Zeiss Confocal microscope.

Electrophysiology

Synaptic activity was recorded from hippocampal pyramidal cells by a whole-cell voltage clamp technique. Data were acquired with an Axopatch 200B amplifier and Clampex 8.0 software (Axon Instruments). Recordings were filtered at 2 kHz and sampled at 200 μs. Tyrode solution was identical as previously described [27], and a hypertonic Tyrode solution was made by adding 500 mM sucrose. The pipette internal solution contained (in mM): 115 Cs-$MeSO_3$, 10 CsCl, 5 NaCl, 10 HEPES, 0.6 EGTA, 20 TEA-Cl, 4 Mg-ATP, 0.3 Na_3GTP (pH 7.35) (300 mOsm). The pipette solution for field stimulation also contained 10 mM QX-314. Field stimulation was applied through parallel platinum electrodes immersed in the perfusion chamber delivering 20 mA pulses.

Fluorescence Imaging

Synaptic boutons were loaded with FM1-43 during a 90 s incubation in Tyrode solution containing 47 mM K^+. After washing with a dye-free Tyrode solution for 10 min, synaptic terminals were destained with a 90 mM K^+ Tyrode solution. All staining and washing solutions contained 10 μM CNQX and 50 μM AP-5 to prevent recurrent activity. Isolated boutons were selected during the wash and fluorescence changes were measured during destaining. Images were obtained by a cooled, intensified digital CCD camera (Roper Scientific) during illumination (1 Hz and 40 ms) at 480 nm via an optical switch (Sutter Instruments). Images were acquired and analyzed with Axon Imaging software (Axon Instruments).

Lentivirus Production

HEK 293 cells were transfected with the Fugene 6 transfection system (Roche Molecular Biochemicals) with the expression plasmid, pFUGW or pFUGW-Cre, and two helper plasmids, delta 8.9 and vesicular stomatitis virus G protein, at 3 μg of each DNA per 75 cm^2 flask [28]. After 48 hr, lentivirus containing culture medium was harvested, filtered at a 0.45 μm pore size, and immediately used for infection. Hippocampal cultures were infected at 7 DIV by adding 300 μl of viral suspension to each well, and recordings were done 13–14 DIV. Titer was determined by counting the number of infected neurons per coverslip (high: >80%; low: <20%).

Statistical Analysis

All data in bar graphs were tested for statistical significance by means of a two-tailed Student's t test. Data included in cumulative histograms were statistically analyzed by the Kolmogorov-Smirnov test.

Supplemental Data

The Supplemental Figure can be found with this article online at http://www.current-biology.com/cgi/content/full/16/7/710/DC1/.

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